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The goal of the intended research was to clone the cDNA for a high affinity choline transport protein and then study its properties. We used several strategies without success in isolating the choline transporter cDNA. Instead, we isolated several other transporter cDNAs and characterized in detail one of them, a cDNA coding for a GABA transporter that is localized to glial cells in the cholinergic electromotor nucleus of Torpedo. In addition to these efforts, we cloned the cDNA for a novel modulator protein for presynaptic calcium channels. We are further characterizing this protein and the other polypeptides with which it interacts under the aegis of a new ARO contract.

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FINAL REPORT

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CAMERON GUNDERSEN, Ph.D.

5 MAY 1993

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C.B. Gundersen

FINAL REPORT

Summary: The goal of this project was to clone the cDNA encoding the high affinity choline transporter of *Torpedo* nerve endings. The expressed protein was to be studied to obtain information germane to the mechanism of solute translocation. Unfortunately, in spite of using numerous approaches (detailed in the full report), we were unable to isolate the choline transporter cDNA. However, using a PCR-based cloning strategy, we obtained several independent *Torpedo* clones that, based on their homology to other Na^+ and Cl^--dependent transporter cDNAs, are likely to encode members of this transporter sub-family. To date, we have identified the solute for only one of these clones. It encodes a GABA transporter that we found to be localized to the glial cells of the purely cholinergic electromotor nucleus of *Torpedo*. In a paper that was just submitted for publication, we speculate on the function of GABA in a CNS nucleus that had no previously known GABAergic component.

In addition to supporting the efforts to clone the cDNA for a choline transporter, we used ARO funds for a parallel investigation to try to isolate cDNAs encoding subunits of presynaptic calcium channels. The justification for this parallel effort is that the original cloning approach for the calcium channel and choline transporter cDNAs was the same. Thus, it proved to be only a small increase in effort to screen for clones encoding components of either of these two crucial synaptic proteins. This work culminated in the isolation of the cDNA for a polypeptide that is essential for the function of presynaptic calcium channels. An ARO contract was awarded to study further this protein, and in particular to seek other polypeptides with which it interacts. This work is in progress at this time.

FINAL REPORT: TEXT

This narrative is divided into three sections that discuss the efforts (i) to isolate a choline transporter cDNA; (ii) to characterize the PCR-derived clone encoding a Na^+ and Cl^--dependent GABA cotransporter and (iii) to identify cDNAs encoding subunits of *Torpedo* presynaptic calcium channels.

(i) Efforts to isolate a choline transporter cDNA from *Torpedo*.

When these studies began, there were no instances in the literature where cDNAs encoding Na^+ and Cl^--dependent co-transporters had been isolated. We had proposed to use an expression cloning approach employing frog oocytes and mRNA from *Torpedo*

electromotor nucleus to isolate a choline transporter cDNA. The rationale for this is that the electromotor neurons should harbor one of the highest abundances of choline transporter mRNA in the animal kingdom, and secondly, several groups had shown that frog oocytes were well-suited for cloning cDNAs for membrane transport proteins. However, in spite of its early promise, this approach was unsuccessful and we turned to several other strategies to try to clone the cDNA for a choline transporter. I will summarize briefly some of the other approaches that we used and indicate where progress may still be made in the future.

The first deviation that we made from the expression cloning approach was motivated by the isolation (by a group in Japan) of a cDNA encoding a yeast choline transporter. Although the yeast choline transporter was <u>not</u> Na^+ of Cl^--dependent, it was a high affinity transporter. Moreover, the cloning strategy that had been used employed a strain of yeast that had been mutated so that their endogenous choline transport capability was eliminated. This gave us two possible paths to search for the *Torpedo* choline transporter. First, we performed Northern and Southern analysis (at low stringency) to test whether there was a yeast homolog of this choline transporter in fish. There was no detectable signal under these circumstances. Second, we used the choline-transporter deficient yeast, and transfected them with *Torpedo* cDNA that should have harbored *Torpedo* choline transporter cDNA. If any mutant yeast had functionally expressed the *Torpedo* clone, then they should have been competent to take up choline and grow in a medium with Na^+ and Cl^- in it. We failed to obtain any such rescues using *Torpedo* cDNA. Thus, both of these "yeast-based" strategies were unsuccessful.

In 1990, Guastella and colleagues reported the isolation of the first cDNA encoding

a Na^+ and Cl^--dependent co-transport protein. Shortly after this seminal report, Pacholczyck and co-workers isolated the cDNA for a second member of this co-transporter family. The sequences for the original rat GABA transporter and human norepinephrine transporter were remarkably similar, particularly in their putative transmembrane domains. This nucleotide and amino acid homology prompted us (and several other groups) to use PCR-based approaches to screen for other cDNAs that had sequence homology to the two original Na^+ and Cl^--dependent transporter clones. Using oligonucleotide primers based on the sequence of the second, fourth and fifth transmembrane domains, we generated DNA fragments of the predicted size for use as probes of a cDNA library. With these PCR-derived probes, we isolated 17 clones from a lambda ZapII library with 2×10^5 recombinants of electric lobe cDNA. Of these 17 clones, 12 were identical and encoded a GABA transporter. Another four clones encoded the same, as yet unidentified transporter, that, though expressed in electric lobe, is much more abundant in electric organ. The final clone encodes a relatively low abundance mRNA that is preferentially seen in nervous tissue and for which we still lack information concerning its substrate. However, neither of these two unidentified clones appears to encode a choline transporter. Moreover, in spite of considerable effort by others, and the mistaken identification by a German group of a cDNA as one that encodes a choline transporter, no one has yet isolated the cDNA that codes for this protein. We are persisting in our efforts using *Torpedo* cDNA to identify this clone, but most of our attention has been focused on the GABA transporter and the calcium channel projects discussed next.

(ii) Characterization of a *Torpedo* GABA transporter.

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Most of this work was done by Geoff Swanson as part of his Ph.D. dissertation project. Numerous cDNAs were isolated from a *Torpedo* library using PCR-derived probes. A sizeable proportion of these cDNAs encoded a GABA transporter. The identity of the substrate of this transporter was established by two means. First, nucleotide sequence analysis revealed that the rat GABA transporter cDNA (encoding GAT-1) was more than 80% identical to the sequence that we obtained for the fish cDNA. When sense cRNA from the fish clone was injected into frog oocytes, we could detect the expression of a GABA transporter in these cells. We used both electrophysiological assays of transporter-generated current and isotopic flux measurements (with [^{3}H]GABA) to characterize this protein. Both its kinetic properties and pharmacology were very similar to the rat GAT-1 clone isolated by Guastella and collaborators. These observations, along with the strong nucleotide and predicted amino acid sequence similarity led us to conclude that we had isolated the fish homolog of the rat GAT-1 GABA transporter. Curiously, the one result that was distinctly different from data obtained for the rat GAT-1 protein, is that the fish protein is located predominantly, if not exclusively, in glial cells of the *Torpedo* electromotor nucleus. By contrast, the rat GAT-1 GABA transporter is generally restricted to neurons, with only a couple of exceptions.

Our discovery of the unexpected glial localization of a GABA transporter in a nucleus that harbors only the cell bodies of cholinergic neurons posed a conundrum. Besides the cholinergic neurons and glial cells, the only other elements in the electric lobe are the afferent axons (and their terminals) and vascular tissue. The afferent input to the electromotor neurons is purely excitatory and there is no known GABAergic input into this nucleus. Thus, we concluded that there must be some hitherto unexplained modulatory role

of GABA in this tissue. Future investigations may shed some light on the conjectural proposals for GABA-mediated activity that we made in our recently submitted manuscript (Swanson et al., 1993).

(iii) Presynaptic Ca channels.

Concomitant with our efforts to use expression cloning to find the cDNA encoding the high affinity choline transporter, we also sought cDNAs coding for functionally important subunits of presynaptic calcium channels. Ultimately, as described in our publication (Gundersen & Umbach, 1992), we used a novel variation of the expression cloning strategy to isolate the cDNA for a small (predicted mass of about 22kDa) protein that is essential for the expression in frog oocytes of omega-conotoxin-sensitive, dihydropyridine resistant (N-type) calcium channels. The physiological and pharmacological characteristics of this channel are appropriate for it to be a presynaptic calcium channel.

This small protein, that we hypothesized is either an essential subunit or modulator of presynaptic calcium channels is expressed only in *Torpedo* nervous tissue, and its mRNA is not detectably seen in liver, muscle or electric organ. This distribution is compatible with this protein being affiliated with presynaptic calcium channels. More recently, using antibodies against this protein, we have confirmed that this protein is found in abundantly innervated tissue (brain, electric organ) but not in liver. Moreover, this protein is membrane-associated, and we are currently using antibodies to map out its membrane topology. A paper describing some of this antibody work is being considered for publication (Mastrogiacomo, Evans & Gundersen, 1993). We expect that the antibodies we have in hand will be very useful for the further characterization of the biochemistry and physiology

of this protein.

PUBLICATIONS

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PARTICIPATING PERSONNEL

1) Cameron Gundersen, Ph.D. (P.I.)

2) Joy Umbach, Ph.D.

3) Geoffrey Swanson: obtained Ph.D. while associated with this project.

4) Alessandro Mastrogiacomo: Post-doctoral fellow; degree is Italian equivalent of Ph.D.

INVENTIONS

None

BIBLIOGRAPHY

None

APPENDICES

None